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Author manuscript

*J Evol Biol.* Author manuscript; available in PMC 2017 April 01.

Published in final edited form as:

*J Evol Biol.* 2016 April ; 29(4): 865–869. doi:10.1111/jeb.12818.

## Hybrid female mate choice as a species isolating mechanism: environment matters

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### Abstract

A fundamental goal of biology is to understand how new species arise and are maintained. Female mate choice is potentially critical to the speciation process: mate choice can prevent hybridization and thereby generate reproductive isolation between potentially interbreeding groups. Yet, in systems where hybridization occurs, mate choice by hybrid females might also play a key role in reproductive isolation by affecting hybrid fitness and contributing to patterns of gene flow between species. We evaluated whether hybrid mate choice behavior could serve as such an isolating mechanism by using spadefoot toad hybrids of *Spea multiplicata* and *S. bombifrons*. We assessed the mate preferences of female hybrid spadefoot toads for sterile hybrid males versus pure-species males in two alternative habitat types in which spadefoots breed: deep or shallow water. We found that, in deep water, hybrid females preferred the calls of sterile hybrid males to those of *S. multiplicata* males. Thus, maladaptive hybrid mate preferences could serve as an isolating mechanism. However, in shallow water, the preference for hybrid male calls was not expressed. Moreover, hybrid females did not prefer hybrid calls to those of *S. bombifrons* in either environment. Because hybrid female mate choice was context-dependent, its efficacy as a reproductive isolating mechanism will depend on both the environment in which females choose their mates as well as the relative frequencies of males in a given population. Thus, reproductive isolation between species, as well as habitat specific patterns of gene flow between species, might depend critically on the nature of hybrid mate preferences and the way in which they vary across environments.

### Keywords

reinforcement; speciation; introgression; sexual selection; condition- and context-dependent mate choice

## INTRODUCTION

A central goal of biology is to understand how new species arise and remain distinct (Coyne & Orr, 2004, Price, 2008, Grant & Grant, 2008, Pfennig & Pfennig, 2012). Under the biological species concept, species are defined as evolutionarily distinct groups that do not exchange genes because they have evolved traits—‘isolating mechanisms’—that prevent

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gene flow between them (Mayr, 1963, reviewed in Coyne & Orr, 2004). A major class of these isolating mechanisms consists of maladaptive traits in hybrids that prevent them from backcrossing to either parent population (Barton & Hewitt, 1985, Coyne & Orr, 2004). Hybrid maladaptation therefore plays a key role in speciation. Consequently, identifying the causes of such maladaptation is crucial to understanding the origins and maintenance of biodiversity (reviewed in Coyne & Orr, 2004).

Historically, speciation research has concentrated on three main sources of hybrid maladaptation: decreased survival, reduced fertility, and decreased likelihood of succeeding in either parental niche (Barton & Hewitt, 1985, Arnold, 1997, Coyne & Orr, 2004, Nosil, 2012, Abbott et al., 2013). A further possibility that has received relatively less attention is that hybrids might express maladaptive reproductive traits that contribute to reproductive isolation between species (Noor, 1997, Russell & Magurran, 2006, Svedin et al., 2008, Clark et al., 2010, Lemmon & Lemmon, 2010, Latour et al., 2014). Specifically, viable, fertile hybrids might fail to appropriately produce or respond to courtship signals (Noor, 1997, Russell & Magurran, 2006, Svedin et al., 2008, Clark et al., 2010, Lemmon & Lemmon, 2010, Latour et al., 2014). Alternatively, hybrids might express mate preferences that reduce their likelihood of mating with fitness-enhancing mates; for example, hybrids might possess intermediate preferences for hybrid males (Hoy et al., 1977, Doherty & Gerhardt, 1983, Ritchie, 2000, Selz et al., 2014) that are sterile or otherwise poor quality mates. In extreme cases, such dysfunctional reproductive behavior could render viable, fertile hybrids 'behaviorally sterile', thereby acting as a key isolating mechanism between species (Noor, 1997, Russell & Magurran, 2006).

In systems where hybrids reproduce with parental species, mating behaviors that influence to which parental species hybrids mate will determine patterns of gene flow between species, including whether such gene flow is directional (Christophe & Baudoin, 1998, den Hartog et al., 2010, Charpentier et al., 2012, Veen et al., 2012, Culumber et al., 2014, Latour et al., 2014, Paczolt et al., 2015). Moreover, because mate choice can depend on the environment or an individual's condition (Cotton et al., 2006), the impact of hybrid mate choice on the extent and pattern of introgression between species could vary in space or time. Thus, evaluating hybrid mate choice and whether it varies across different contexts is critical for explaining reproductive isolation and patterns of gene exchange, if any, between species (Rosenthal, 2013).

We addressed these issues using hybrid female spadefoot toads derived from crosses between *Spea multiplicata* and *S. bombifrons*. As described below, this system is well suited for evaluating hybrid female preferences for sterile hybrid males versus pure-species males across different environments.

## MATERIALS AND METHODS

### Study System

We studied first-generation ( $F_1$ ) hybrid females of two spadefoot toads: *Spea bombifrons* and *S. multiplicata*. These species hybridize in the southwestern USA and northern Mexico (Pfennig et al., 2012). Hybrids are viable; however,  $F_1$  hybrid females produce half as many

eggs as pure-species females whereas F<sub>1</sub> hybrid males are sterile (Simovich et al., 1991, Wünsch & Pfennig, 2013). Hybrid males attempt to attract mates, but their calls are intermediate between those of pure-species males (Pfennig, 2000). If hybrid females possess intermediate preferences for sterile males, this could lead to selection disfavoring hybrid females.

Female *S. multiplicata* do not obtain fitness benefits by hybridizing with *S. bombifrons* (Pfennig & Simovich, 2002), and avoid hybridizing where the two species co-occur (Pfennig, 2000, Pfennig & Rice, 2014). By contrast, *S. bombifrons* females can benefit by hybridizing, but only in shallow, rapidly drying ponds (Pfennig & Simovich, 2002, Pfennig, 2007). Specifically, hybrid tadpoles develop faster than pure *S. bombifrons* tadpoles, so hybrids are more likely to reach metamorphosis and therefore survive in shallow, highly ephemeral pools (Pfennig & Simovich, 2002, Pfennig, 2007). By contrast, in deep, long-lasting ponds, *S. bombifrons* females receive no such benefit because *S. bombifrons* tadpoles can escape the ponds (Pfennig & Simovich, 2002, Pfennig, 2007). In conjunction with these environmentally dependent fitness consequences of hybridization, *S. bombifrons* females have evolved facultative preferences for conspecifics. They prefer conspecific males in deep, long lasting pools, but switch their preferences and are more likely to prefer *S. multiplicata* males in shallow, ephemeral pools (Pfennig, 2007). Such plasticity in mate preferences could be inherited by hybrid females and impact their mate choice decisions.

### Phonotaxis Tests

We tested the mate preferences of 20 gravid, lab-bred F<sub>1</sub> hybrid females. The females were derived from 12 families (i.e., some females were siblings). For 10 females *S. bombifrons* was maternal, and for the other 10 females *S. multiplicata* was maternal.

Each female was presented with the following pairwise choices of male call stimuli: F<sub>1</sub> hybrid calls versus *S. multiplicata* calls; F<sub>1</sub> hybrid calls versus *S. bombifrons* calls; and *S. bombifrons* calls versus *S. multiplicata* calls. For each stimulus combination, each female was tested four times in deep water and four times in shallow water. The order in which females were presented the call pairings and water level was random.

We generated the call stimuli using Audacity (a free sound editor software). Spadefoots produce simple, pulsatile calls, so we synthesized our call stimuli by generating pulses and building call series from those. Specifically, for each stimulus type (i.e., hybrid, *S. bombifrons* or *S. multiplicata*), we first modified a pure tone to create a triangular pulse. We then repeated the pulse at the rate and for the call duration of each stimulus type to produce a single call for each stimulus type. Using these individual calls, we next generated a 30-second call series for each stimulus type by repeating these calls, interspersed with silence (this inter-call silence interval was constant across the call series). Call frequency, pulse rate within the call, call duration, and call rate during the 30 second series consisted of approximately mean call properties for sympatric *S. multiplicata*, *S. bombifrons*, and F<sub>1</sub> hybrids from southeastern AZ (Pfennig, 2000, Pfennig, 2007).

We measured female mate preferences using previously published methods (Pfennig, 2007). Specifically, we placed each toad in the center of a circular wading pool 1.8 m in diameter

filled approximately to 30 cm (deep water) or 6 cm (shallow water). Each toad was initially placed on a central platform 2 cm above water level, equidistant between two platforms set 180° apart at the edges of the pool. We placed a speaker on each of these two platforms. Two additional platforms were set at 90° from the speakers to serve as neutral areas.

At the start of each trial, we placed each female under an opaque container on the central platform for an acclimation period of 5 minutes. The call stimuli began playing antiphonally at the start of this period. At the end of the acclimation interval, we released the female and she was allowed to move freely around the arena while the stimuli continued to play. Females were watched continuously via an infrared camera by an observer in a different room.

When the female moved within one body length of, or touched, a speaker, she was scored as preferring that stimulus. We recorded the time taken for females to touch the speaker as a female's latency to choose; for each female we generated a mean latency value for each stimulus combination and for each water level. If a female did not touch a speaker within 30 minutes, she was considered non-responsive in that trial.

The call stimuli were switched between speakers after each female was tested to control for side biases. Moreover, in each stimulus pairing, the stimulus type each female heard first was randomly determined to control for any possible leader/follower effects.

## Statistical Analyses

We determined whether females differed in their responsiveness to male call pairings or to the different water levels by contrasting latency time to choose for these variables using Wilcoxon/Kruskal-Wallis rank sums tests. We also contrasted the overall mean time hybrid females took to choose a stimulus with the mean time measured previously for pure species females. To do so, we used a Wilcoxon signed-rank test to determine if the overall mean for hybrids differed from the hypothesized mean of 439 seconds, which is a previously measured combined mean to choose between conspecific and heterospecific calls across different water levels for *S. multiplicata* and *S. bombifrons* females (Pfennig, 2007).

We evaluated female preferences for each stimulus pair in deep and shallow water. To do so, we used Wilcoxon tests to determine whether females significantly preferred one stimulus versus the other in a given water level. Female preferences in each water level were measured as the proportion of times that they chose the hybrid stimulus (in the hybrid versus parent species tests) or the proportion of times they chose the *S. bombifrons* stimulus (in the *S. bombifrons* versus *S. multiplicata* trials). Random expectation for these preferences was 50%. To determine if water level affected female preferences in the one stimulus combination where preferences were detected (hybrids versus *S. multiplicata*; see Results), we used a Wilcoxon test to contrast the mean preferences (as measured above) in deep versus shallow water. Our alpha level for all analyses was 0.05. Our data are available as a supplementary data file.

## RESULTS

We found that hybrid females did not differ in the time taken to choose a stimulus depending on either the call pairings (rank sums test, chi-square = 1.22,  $df = 2$ ,  $P = 0.545$ ) or on water level (rank sums test, chi-square = 0.21,  $df = 1$ ,  $P = 0.646$ ). However, the overall mean response time ( $\pm$  SD) of hybrid females, 385.2 (98.33) sec. was faster than the hypothesized mean of 439 sec. derived from a previous study in which pure-species females were presented conspecific versus heterospecific calls across the two water levels (Wilcoxon signed rank = -57;  $df = 19$ ,  $P = 0.033$ ).

In our analyses of each call stimulus pairing, we found that hybrid females expressed a significant, non-random preference in only one pairing (Table 1). Specifically, in deep water, hybrid females preferred hybrid calls when given a choice between hybrid calls and *S. multiplicata* calls (Table 1). This preference for hybrid males was not expressed in low water (Table 1; Mean difference (SD) % of times hybrid chosen in deep water– % of times hybrid chosen in low water = 15.85% (33.16); Wilcoxon signed-rank = 40,  $df = 19$ ;  $P = 0.058$ ). Thus, hybrid females appear to show no preference for male types except when choosing between hybrid and *S. multiplicata* males in deep water.

## DISCUSSION

We evaluated hybrid female preferences for the calls of pure-species males and sterile hybrid males. Hybrid females did not express a significant preference for any particular male type except in deep water. In the deep-water environment, spadefoot toad hybrid females preferred the calls of sterile hybrid males versus those of *S. multiplicata*, indicating that hybrid female mate preferences could be maladaptive in at least some circumstances. Spadefoot females breed no more than once per year, so choosing a sterile mate carries severe lifetime fitness costs. Critically, such behavior would lower the incidence of backcrossing to either parent species, and therefore reduce gene flow between the two species. Consistent with our findings, a previously published survey of natural pairs across several populations showed that, of 82 pairs involving hybrid females, 34 (i.e., 41%) were with hybrid males, even though hybrid males represented only 15% of mated males (Pfennig & Simovich, 2002). This study did not associate pair types with water level, however, so future work is needed to evaluate how hybrid behavior varies in natural populations depending on the breeding environment.

Generally, the role of hybrid behavior as a reproductive isolating mechanism has been underappreciated relative to studies of hybrid sterility, inviability, or ecological performance (Rosenthal, 2013). Nevertheless, our results comport with an emerging body of evidence (e.g., Noor, 1997, Russell & Magurran, 2006, Svedin et al., 2008, Clark et al., 2010, Lemmon & Lemmon, 2010, Latour et al., 2014), which reveals that maladaptive hybrid mating behaviors could contribute to reproductive isolation between species.

Despite finding that hybrid mate preferences can potentially serve as an isolating barrier in at least some conditions, our results also reveal that hybrid mate choice depends on a female's environment. Female mate choice is often context- or condition-dependent (Cotton

et al., 2006). In the case of spadefoots, hybrid females did not switch their mate preferences from one male type to another, as occurs in pure-species *S. bombifrons* females (Pfennig, 2007). Instead, hybrid females as a group appear to be less choosy depending on habitat type or males that are encountered.

Generally, the possibility that female hybrids in a given system might vary their mate choice behavior has two key implications. First, whether hybrid mate choice is an effective isolating mechanism will depend on the environment. Second, patterns of hybrid mate choice (and how they vary with the environment) can impact the directionality, if any, of introgression between species (Christophe & Baudoin, 1998, den Hartog et al., 2010, Charpentier et al., 2012, Veen et al., 2012, Culumber et al., 2014, Latour et al., 2014, Paczolt et al., 2015). The expression of alternative preferences by hybrid females across different habitats could generate habitat-dependent patterns of introgression that are linked to female mate preferences. In the absence of understanding how hybrid mate choice varies across habitats, that ultimate cause of environmental variation in introgression could be missed (Rosenthal, 2013).

Moreover, if hybrid females mate randomly, then the relative frequencies of male types in a population can also contribute to mating patterns (sensu Malmos et al., 2001, see also Culumber et al., 2014). Thus, the extent to which relative male abundance dictates patterns of introgression will likely depend on the strength of hybrid female preferences. When hybrid mate preferences are weakly expressed (e.g., as in the spadefoots in shallow water habitats), the relative frequencies of different male types might be more important to reproductive isolation – or lack thereof – than when mate preferences are stronger (e.g., as in the spadefoots in deep water habitats), especially if females reject non-preferred males. In spadefoots, findings from the survey of natural pairs mentioned above are consistent with this possibility. Hybrid females were more often mated to *S. multiplicata* males than to *S. bombifrons* males across populations where *S. bombifrons* males constituted only 10% of mated males (Pfennig & Simovich, 2002, see also Simovich, 1985). Indeed, backcross offspring to *S. multiplicata* are more frequent than backcross offspring to *S. bombifrons* (Pfennig & Simovich, 2002). Hybrid males are sterile, so backcrossing is mediated by hybrid female behavior.

From evolutionary and ecological perspectives, understanding speciation requires determining under what environmental circumstances reproductive isolation evolves and is either maintained or breaks down. Hybrid mate preferences will potentially play a key role in this process depending on how those preferences vary with the environment and the relative abundance of pure-species and hybrid males. Thus, evaluating how these different factors combine is a critical next step to ascertaining the role of hybrid reproductive behavior in the origins and maintenance of species.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



## ACKNOWLEDGEMENTS

We are grateful to members of K. Pfennig's lab for assistance with observations. Many thanks also to D. Pfennig, B. Stuart, N. Levis, S. De La Serna Buzon, A. Serrato, M. Servedio, J. Kingsolver, S. Burmeister, two anonymous reviewers, and M. Ritchie for comments that improved the manuscript. A New Innovator Award from the Office of The Director, National Institutes of Health (1 DP2 OD004436-01) to KSP funded the work. UNC's IACUC approved all procedures.

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**Table 1**

Mean hybrid female preferences for alternative call stimuli in either deep (D) or shallow (S) water. Females were presented pairwise call stimuli of *S. bombifrons* (B), *S. multiplicata* (M) or F<sub>1</sub> hybrid (H) males. Preferences are presented as percent *S. bombifrons* (in B v M trials) or percent hybrids (in B v H and M v H) chosen across repeated presentation of a given stimulus set.  $df = 19$  for all tests; random expectation is 50%.

Call Stimuli Pair (Water level)	Mean preference (SD), %	Wilcoxon signed rank (P-value)
B v M (D)	57.1 (23.49)	18.5 (0.27)
B v M (S)	41.62 (31.20)	-17.0 (0.39)
H v B (D)	48.35 (23.86)	-3.5 (0.79)
H v B (S)	50.85 (27.17)	0.0 (1.00)
H v M (D)	64.19 (23.28)	<b>37.5 (0.03)</b>
H v M (S)	48.34 (25.78)	-10.0 (0.67)